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Pygopus and Legless Provide Essential Transcriptional Coactivator Functions to Armadillo/β-Catenin

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Summary

Wnt signaling controls important aspects of animal development, and its deregulation has been causally linked to cancer. Transduction of Wnt signals entails the association of β-catenin with nuclear TCF DNA binding proteins and the subsequent activation of target genes. The transcriptional activity of Armadillo (Arm, the *Drosophila* β-catenin homolog) largely depends on two recently discovered components, Legless (Lgs) and Pygopus (Pygo). Lgs functions as an adaptor between Arm/β-catenin and Pygo, but different mechanisms have been proposed as to how Arm/β-catenin is controlled by Lgs and Pygo. Although Lgs and Pygo were originally thought to serve as nuclear cofactors for Arm/β-catenin to enhance its transactivation capacity, a recent analysis argued that they function instead to target Arm/β-catenin to the nucleus. Here, we used genetic assays in cultured cells and in vivo to discriminate between the two paradigms. Regardless of the measures taken to maintain the nuclear presence of Arm/β-catenin, a transcriptional-activation function of Pygo could not be bypassed. Our findings therefore indicate that Arm/β-catenin depends on Lgs and Pygo primarily for its transcriptional output rather than for its nuclear import.

Results and Discussion

Wingless/Int (Wnt) signals are secreted glycoproteins controlling many fundamental processes during animal development [1]. Whereas several responses to Wnt ligands appear to entail direct cytoplasmic responses organizing planar cell polarity and organ morphogenesis [2, 3], a significant fraction of Wnt responses concern transcriptional changes in the nucleus [4]. This latter aspect of Wnt-signal transduction is mediated by β-catenin and is often referred to as "canonical" or β-catenin-dependent Wnt signaling. The canonical Wnt pathway plays important roles in embryonic-cell-fate determination, and its constitutive activation is oncogenic in several adult mammalian tissues, most notably in the intestinal epithelium [5, 6]. Hence, it is of prime interest to understand how β-catenin activity can upregulate transcription of Wnt target genes. Although cytoplasmic β-catenin was originally discovered through its role in cell adhesion, a large body of evidence indicates that it is degraded in the absence of a Wnt signal but stabilized in its presence. As a consequence, β-catenin can sufficiently accumulate, translocate to the nucleus, and be directed to Wnt target genes by associating with DNA-binding TCF/LEF proteins (reviewed in [7, 8]). However, it is less clear how a cell-adhesion component, relocated to the nucleus, can promote and sustain the transcriptional activity of these targets.

Using genetic assays in *Drosophila*, we have recently identified a presumptive adaptor protein, Legless (Lgs) [9], that binds to β-catenin and its *Drosophila* homolog, Armadillo (Arm), as well as to the nuclear protein Pygopus (Pygo) [9–12]. On the basis of biochemical and phenotypic analysis, we proposed that nuclear β-catenin/Arm assembles a quaternary complex, consisting of TCF, β-catenin, Lgs, and Pygo, in which Pygo serves as a transcriptional activator to induce and/or maintain the transcription of Wnt/Wg target genes [9]. Alternatively, however, the requirement for Lgs and Pygo in Wnt/Wg signaling could be attributed to a role in targeting and retaining β-catenin in the nucleus, increasing its net nuclear concentration and, hence, its activity. This latter view has recently gained recognition [13] and experimental support [14] by a cell-biological analysis of these components. Here, we set out to address the mechanistic role of Pygo by subjecting the two models to three different tests and come, in each case, to the conclusion that Pygo functions mainly in the transcriptional output of β-catenin.

In the first approach, we examined the consequences of disrupting the molecular interaction between β-catenin and Lgs. We have recently identified β-catenin/Arm amino acid residues required for Lgs binding and observed that mutant β-catenin forms lacking these residues are severely compromised in their signaling activity [15]. This reduction in activity could be caused either by a failure of β-catenin/Arm to recruit the "transcriptional mediator" Pygo or by a reduced (as a result of diminished nuclear anchoring) nuclear-cytoplasmic ratio of β-catenin/Arm. We repeated an experiment in which N-terminally truncated and therefore constitutively active forms of Arm, Arm^{S10}-wt and Arm^{S10}-D164A (differing solely in one critical amino acid residue necessary for Lgs binding), were expressed in the embryonic epidermis of *Drosophila* [15, 16]. Expression of Arm^{S10}-wt suppressed denticle formation (Figure 1B)—a read-out for a gain of Wg signaling activity—whereas the D164A mutation, which impairs binding to Lgs, efficiently abolished this gain-of-function activity (Figure 1C and [15]). When subjected to an immunohistochemical analysis, however, the two genotypes visually differed neither in amount nor subcellular localization of the Arm^{S10} proteins (Figures 1A′–1C′). We further used the D164A mutation in a cellular assay in which a constitutively active form of β-catenin (S33Y) was tethered to the enhancer of a reporter gene by the DNA binding domain of Gal4. Whereas β-catenin caused strong transcriptional acti-

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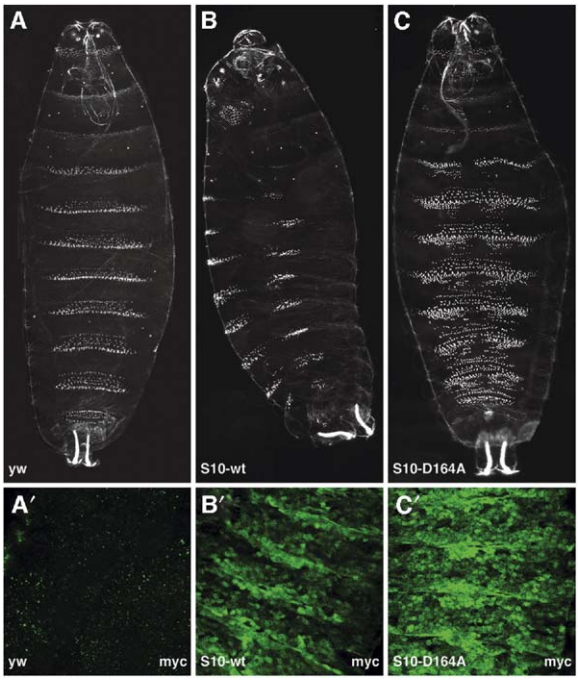

Figure 1. Constitutively Active Forms of Arm Depend on Lgs Binding for Signaling But Not for Localization

(A–C) Cuticles of embryos with different genotypes are shown. (A) Wild-type embryos show a repeated pattern of denticle belts on their ventral surface. (B) Ubiquitous expression of a constitutively active form of Arm (Arm^{S10}-wt) results in a naked-cuticle phenotype, a hallmark of ectopic Wg signaling activity [16, 27]. (C) Ubiquitous expression of a constitutively active Arm form impaired for Lgs binding (Arm^{S10}-D164A) no longer causes a naked-cuticle phenotype; indeed, a slight dominant-negative effect can be observed (ectopic denticles between denticle belts).

(A′–C′) Expression of transgene-derived Arm proteins was revealed by confocal microscopy with an α-myc antibody. (A′) No staining is seen in wild-type embryos. (B′, C′) Arm^{S10} proteins are present in the nucleus and also in the cytoplasm. The localization of Arm^{S10}-D164A cannot be distinguished from that of Arm^{S10}-wt.

All *arm* transgenes in these experiments contain a Myc epitope in their C-terminal region [15, 16] and were controlled by a UAS-promoter driven by *daughterless-Gal4* [28].

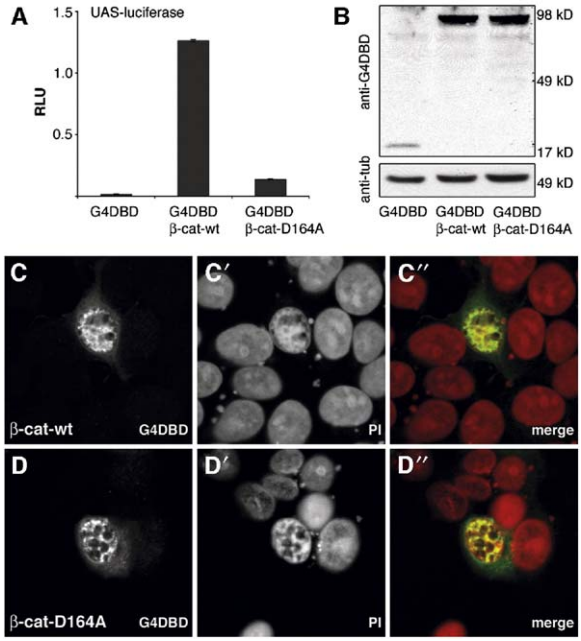

Figure 2. The D164A Mutation, Which Prevents Lgs Binding, Severely Reduces the Transcriptional Activity of β-Catenin without Affecting Subcellular Localization

(A) Relative reporter-gene activities induced by DNA-tethered mouse β-catenin-wt and β-catenin-D164A assayed in 293T cells. Constitutively active β-catenin (S33Y) bound to the Gal4 DNA binding domain (G4DBD-S33Y-β-cat-wt) causes a strong transcriptional activation of the UAS-luciferase reporter, whereas the D164A mutant form has strongly reduced activity. Error bars represent standard deviations of triplicates.

(B) Western blot with 293T cell lysates demonstrates that both forms of β-catenin proteins are expressed at equivalent levels.

(C and D) Immunostaining of transfected 293T cells shows no significant differences in subcellular localization between G4DBD-S33Y-β-cat-wt and G4DBD-S33Y-β-cat-D164A proteins.

(C′ and D′) Propidium iodide (PI) staining to mark cell nuclei.

(C″ and D″) Merge of the panels to the left, with PI staining in red, G4DBD in green.

We obtained essentially the same results with Gal4-β-catenin fusion proteins that additionally contained an N-terminal nuclear-localization signal (NLS-G4DBD-S33Y-β-cat-wt and NLS-G4DBD-S33Y-β-cat-D164A).

vation, the D164A form lost this activity almost completely (Figure 2A). Importantly, however, both forms were expressed at equivalent levels in human cells (Figure 2B) and did not differ in their ability to localize in nuclei (Figures 2C and 2D). Because Lgs mediates the binding of β-catenin to Pygo [9, 17], we interpret these results as evidence that a failure of Arm/β-catenin to recruit Pygo impedes the transcriptional activity of the former despite the fact that it is nuclearly localized.

A second test was devised on the assumption that Lgs appears to function merely as an adaptor between Arm/β-catenin and Pygo, thereby linking Arm/β-catenin either to a transcriptional activator or a nuclear anchor. Such a passive role for Lgs can be inferred from the observations that Lgs is dependent on Pygo both for its signaling activity [9] and for its nuclear localization [14]. If the main role of Lgs would be to link Arm/β-catenin to the constitutively nuclear anchor Pygo, it should gain functional independence of Pygo when bestowed with a nuclear-localization signal (NLS). We therefore modified Lgs by replacing a C-terminal portion with sequences of a green fluorescent protein (LgsN-eGFP) and adding the NLS of SV40 large T-antigen N-terminally (NLS-LgsN-eGFP). These altered forms of Lgs were examined for their subcellular distribution and signaling function. The addition of a single NLS effectively conferred nuclear localization, as assessed in transfected cells (Figures 3A–3D). When tested for their signaling capacity in *Drosophila* S2 cells, LgsN and NLS-LgsN were found to be equally active in rescuing the RNAi-mediated knockdown of endogenous Lgs (Figure 3E). However, these two forms of Lgs were equally inactive in rescuing the knockdown of endogenous Pygo. Consistent with this result, we also found that the Lgs-rescuing activity of NLS-LgsN still depends on the HD1 domain [9], through which it binds Pygo (Figure

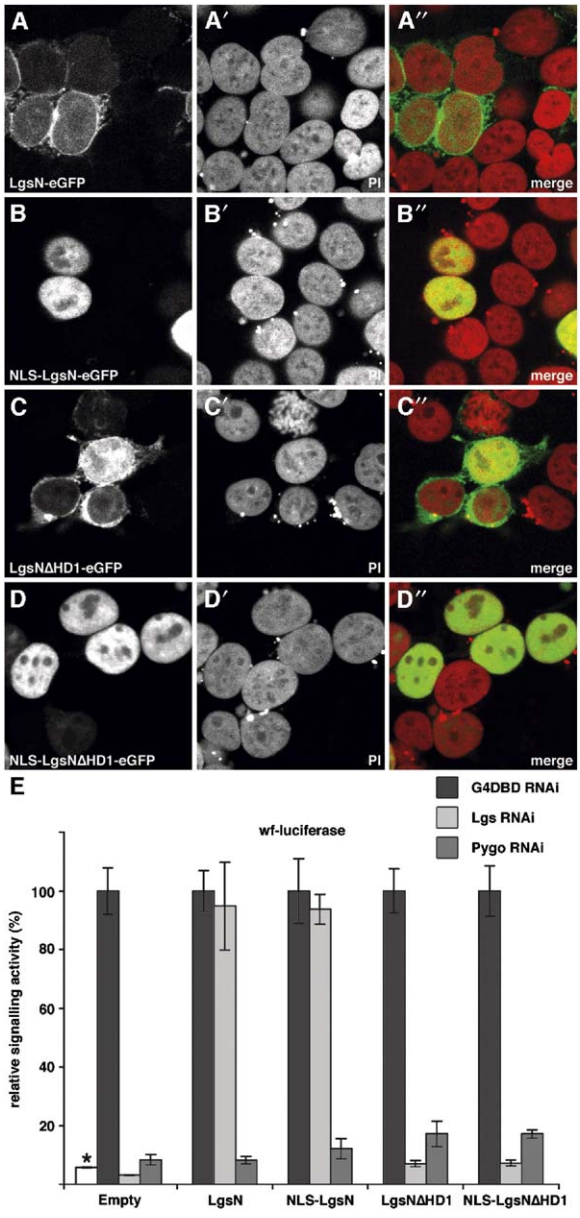

Figure 3. Nuclearly Targeted LgsN Can Rescue the Knockdown of *lgs* But Not of *pygo* in a Cell-Based Wg Signaling Assay

(A–D) Localization of the different forms of Lgs in 293T cells. All forms contain eGFP instead of the C-terminal amino acids 783 to 1464. An even shorter version of Lgs (amino acids 1–583) has been shown to rescue *lgs* mutant animals [9]. Most LgsN and LgsNΔHD1 protein is found in the cytoplasm (except for a small percentage of cells in which LgsNΔHD1 can be seen all over the cell). NLS-LgsN and NLS-LgsNΔHD1, however, are exclusively nuclear.

(A′–D′) Propidium iodide (PI) staining to mark cell nuclei.

(A″–D″) Merge of the panels to the left, PI staining in red, eGPF in green. The same results were obtained in HeLa cells (data not shown).

(E) Signaling capacities of different forms of Lgs (eGFP-tagged) are shown in the presence or absence of endogenous Lgs and Pygo. LgsN was used here instead of full-length Lgs to allow selective knockdown of endogenous Lgs by means of RNAi directed against the 3′ half of the *lgs* transcript. *Drosophila* S2 cells were transfected with the *wf-luc* reporter construct (see Experimental Procedures), expression plasmids as indicated underneath the bar diagram, and were treated with dsRNA as indicated in the legend. Cells were cocultured with Wg-secreting cells [25], resulting in a 20-fold stimulation of *luciferase* expression. All bars are from cells in the Wg-induced state except the asterisk-marked one, which represents the uninduced state. Rescuing activities of the different forms of Lgs are represented relative to the respective G4DBD negative control RNAi (set to 100%). LgsN can rescue *lgs* RNAi but not *pygo* RNAi. The same was observed for NLS-LgsN. Neither LgsNΔHD1 nor NLS-LgsNΔHD1 can rescue *lgs* or *pygo* RNAi, indicating that the signaling capacities of LgsN and NLS-LgsN depend on Pygo binding. Error bars represent standard deviations of triplicates.

3E). Together, these results indicate that constitutive nuclear targeting of Lgs does not bypass the requirement for Pygo in Wg signaling, suggesting that Pygo must provide a function beyond ensuring availability of Lgs and β-catenin in the nucleus of Wg-transducing cells.

Our third test aimed at assessing the role of the N-terminal homology domain (NHD) of Pygo. *Drosophila* Pygo and its two mammalian homologs, Pygo1 and Pygo2, share—in addition to their C-terminal plant homology domain (PHD) finger domain, through which they bind Lgs—a short N-terminally located sequence of amino acids [9, 12]. On the basis of the conservation of Pygo function and absence of further common domains, the NHD was proposed to serve as transactivation domain [9]. We first confirmed that the NHD core domain (amino acids 91 to 101) is not required for nuclear localization of Pygo because neither the deletion of the core nor the change of a conserved and functionally required amino acid (F99A) affected the nuclear localization of Pygo in cultured cells (data not shown), consistent with the findings of Townsley et al. [18]. Importantly, these alterations also had no discernible effect on the capacity of Pygo to bind Lgs, as shown for Pygo-F99A in Figure 4A. If Pygo and Lgs primarily function to target Arm/β-catenin to the nucleus, then NHD mutations should not seriously affect Wnt/Wg signaling. We found, however, that Pygo-F99A—in contrast to wild-type Pygo—failed to rescue Pygo function in cultured cells and in vivo (R.S. and K.B., unpublished data). We then replaced the endogenous *pygo* gene with a genomic *pygo-F99A* transgene in vivo, and we observed that both mutant and wild-type Pygo proteins were expressed at comparable levels without detectable differences in nuclear-cytoplasmic distribution (Figures 4B–4D). The most explicit argument for a role of the NHD in transactivation was obtained by analyzing mutant clones of imaginal cells in which either the *pygo-wt* or the *pygo-F99A* transgenes were the only source of full-length Pygo protein (Figures 4E–4G). Both transgenes rescued Lgs nuclear localization in the mutant clones to a similar extent; however, *pygo-F99A*—but not *pygo-wt*—showed severely reduced transcription of the Wg target gene *senseless* [19]. Because Pygo protein bearing a mutant NHD retains the capacity to localize Lgs (and, by inference, Arm), we infer that the key function of the Pygo NHD is to confer transcriptional activity to Arm.

In summary, we have tested the function of Lgs and Pygo in β-catenin-dependent Wnt/Wg signaling by devising experiments that separate a role in transcriptional ac-

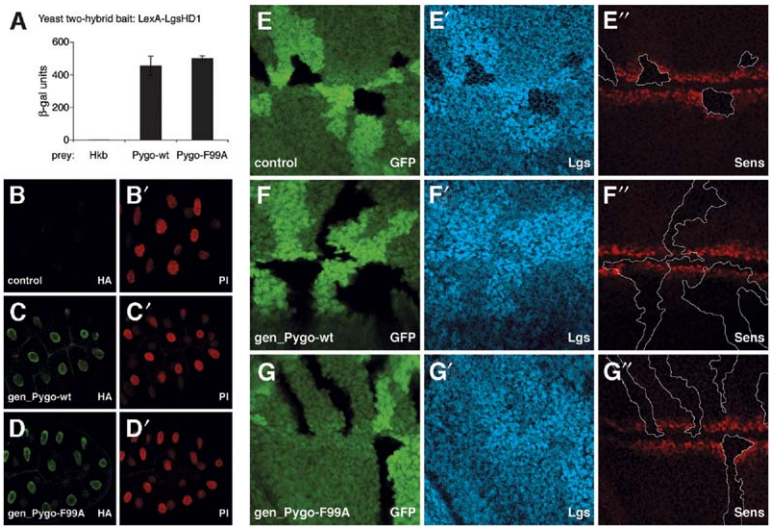

Figure 4. A Single-Point Mutation in the Pygo NHD Affects Neither Lgs Binding Nor Nuclear Localization, But Severely Reduces Its Signaling Ability

(A) A quantitative yeast two-hybrid assay confirms that dPygo-wt and dPygo-F99A both strongly bind to LgsHD1 but not to the negative control Huckebein (Hkb), a transcription factor [29] that plays no role in Wnt/Wg signaling. Error bars represent standard deviations of triplicates.

(B–D) Immunostaining of *Drosophila* salivary glands demonstrates that dPygo-wt and dPygo-F99A localize to the nucleus.

(B′–D′) Propidium iodide (PI) staining to mark cell nuclei.

(E–G) *Drosophila* wing imaginal discs containing cell clones from which endogenous *pygo* function has been eliminated. The clones are marked by the absence of GFP expression (GFP shown in green) and assayed for Lgs distribution (blue) and Senseless (Sens) expression (red). (E) In discs expressing no *pygo* transgene, the removal of *pygo* activity causes cytoplasmic localization of Lgs and a severe reduction of Sens expression. (F) A genomic transgene encoding dPygo-wt partly rescues nuclear Lgs localization and Sens expression in *pygo* mutant tissue. (G) dPygo-F99A can only rescue Lgs localization, but not restore Sens expression in *pygo* clones.

tivation of targets from a role in nuclear targeting or retention of Arm/β-catenin. In all three situations examined, the transcriptional output of Arm/β-catenin depended on Pygo activity despite measures to grant Arm/β-catenin such alleged nuclear retention. When Arm/β-catenin was tethered directly to DNA via the Gal4 DNA binding domain, or when Lgs was endowed with an NLS of its own, Arm/β-catenin activity was still dependent on the recruitment of Pygo. Likewise, in vivo, when the nuclear retention activity of Pygo was left intact, Arm was not able to transduce Wg and activate target genes without the Pygo NHD. Although we cannot rule out that Lgs and Pygo function as a nuclear anchor for β-catenin, our results collectively argue that the primary requirement for the two Arm/β-catenin partners must be attributed to a transcriptional role that allows Arm/β-catenin to activate and/or sustain the expression of Wnt/Wg target genes. Although we presently lack information on the biochemical nature of this transactivation activity, it is tempting to assume that it involves the NHD-mediated recruitment of a chromatin-modification complex or of factors mediating transcription initiation or elongation.

Experimental Procedures

Plasmid Constructs

For the *wf-luc* reporter plasmid, a 2.2 kb fragment of the *wingful/Notum* gene ([20, 21]; 4099 to 1866 bp upstream of ATG) was amplified by polymerase chain reaction (PCR) and inserted into the pGL3 firefly luciferase reporter (Promega). The minimal *SV40* promoter was replaced by a minimal *hsp70* promoter (A. Smith, M. Kuster, R.S., and K.B., unpublished data). The *UAS-luc* reporter plasmid used in 293T cells was generated by inserting the five tandem-arrayed optimized Gal4 binding sites from pUAST [22] into a modified pGL3 vector, where the minimal *SV40* promoter was replaced by a minimal *cFos* promoter. pcDNA3.1 (Invitrogen) or pPacPL [23] vectors were used for expression of cDNAs in mammalian cells or S2 cells, respectively. The LgsN constructs contain amino acids 1–782. In the ΔHD1 constructs, amino acids 318–345 are replaced by a BglII site. For simplicity, we use the amino acid numbering of *Drosophila* Pygo for both human and *Drosophila* Pygo throughout the text. dPygo-F99 would correspond to hPygo2-F78.

Antibodies

The following antibodies were used: rabbit anti-Gal4DBD (SC-577, Santa Cruz), rabbit anti-Myc (A14, Santa Cruz), rat anti-HA (3F10, Roche), mouse anti-Tubulin (B-5-1-2, Sigma), rabbit anti-Lgs [9], guinea pig anti-Senseless [19], goat anti-rabbit conjugated with Alexa 488 (Molecular Probes), goat anti-rat conjugated with fluorescein isothiocyanate (FITC) (Jackson Labs), goat anti-rabbit-HRP (Jackson Labs), goat anti-rabbit conjugated with Cy5 (Jackson Labs), and goat anti-guinea pig conjugated with Alexa 568 (Molecular Probes).

dsRNA Production

Templates for G4DBD, GFP, Lgs, and Pygo double-stranded RNA (dsRNA) synthesis were generated by PCR with the following primers containing 5′ T7 promoter tails:

fG4DBD 5′-T7CTACTGTCTTCTATCGAACAAG-3′, rG4DBD 5′-T7ATACAGTCAACTGTCTTTGAC-3′, product length: 0.43kb;
fGFP 5′-T7CTTTTCACTGGAGTTGTCC-3′, rGFP 5′-T7ATCCATGCCATGTGTAATCC-3′, product length: 0.68kb;
fLgs 5′-T7GGCATGCGTCCACATGCC-3′, rLgs 5′-T7ATTTGTTGACAAAGAACGTTG-3′, product length: 0.58kb;
fPygo 5′-T7TGGTGATGCGGATGTCC-3′, rPygo 5′-T7TCCATGTCCACGTCAGTGC-3′, product length 0.61kb;

dsRNA was then synthesized from these templates with the Ambion Megascript kit. For RNAi in S2 cells [24], 1 μg dsRNA was used per well (96-well plate).

Cell Culture

All transfections were performed with polyethylenimine (PEI, Polysciences). S2-cell transfections were done in 96-well plates with a total of 500 ng DNA per 3 wells (50 ng *wf-luc*, 100 ng *actin5c-renilla*, 50 ng *tubulinα1-dfrizzled2*, 50 ng rescue constructs, and 250 ng empty vector). The Wg signaling pathway was induced 48 hr after transfection by the addition of heat-shocked S2 cells stably transfected with Wg cDNA under the control of a heat-shock promoter as described [25]. 293T cell transfections were performed in 12-well plates with a total of 1.5 ug DNA per 3 wells (500 ng *UAS-luc*, 100 ng pRL-TK (Promega), 500 ng Gal4DBD constructs, and 400 ng of empty vector). Luciferase activities were determined after 72

hr for S2 cells and after 48 hr for 293T cells via the Dual-Luciferase Assay System (Promega).

Immunohistochemistry

For embryonic stainings, embryos were dechorionated in bleach and fixed for 30 min at the interphase of a heptane/8% formaldehyde in PEM (0.1 M Pipes, 1 mM EGTA, and 2 mM $MgSO_4$) solution. The aqueous phase was removed, and an equal amount of methanol was added to devitellinize the embryos. Mutant clones in wing imaginal discs were generated by crossing FRT82 *pygo*^{S130}/TM6b carrying either genomic Pygo-wt or F99A to hsp70-flp; FRT82 ubi-GFP/TM6b. Seventy-two hours after egg laying, larvae were heat shocked at 37.5°C for 45 min. Late third-instar larvae were dissected, and imaginal discs and salivary glands were fixed and stained by standard techniques.

293T cells were fixed in 4% paraformaldehyde 48 hr after transfection.

Yeast Two-Hybrid Assays

The yeast two-hybrid system as described in chapter 3 of Bartel and Fields [26] was used. Interactions between proteins were measured with the quantitative "Liquid Culture Assay Using ONPG as Substrate" (Clontech, Yeast Protocols Handbook, http://www.clontech.com/clontech/techinfo/manuals).

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